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This work is based on the use of the sol-gel process to encapsulate biomolecules in a porous silicate matrix. The porosity of the matrix is such that small molecules and ions can readily diffuse into the materials while the much larger proteins remain trapped in the silicate network. Using this approach, it is possible to develop new types of solid state sensors which use the specificity of enzymes and proteins for binding of substrates or related molecules. The objective of our initial work has been to obtain electron transport from the redox center of an encapsulated enzyme (making use of redox-active molecules or mediators) so as to measure the electrochemical response of the doped sol-gel to an external analyte.

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SOL-GEL BASED ELECTROCHEMICAL BIOSENSORS

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This work is based on the use of the sol-gel process to encapsulate biomolecules in a porous silicate matrix. The porosity of the matrix is such that small molecules and ions can readily diffuse into the materials while the much larger proteins remain trapped in the silicate network. Using this approach, it is possible to develop new types of solid state sensors which use the specificity of enzymes and proteins for binding of substrates or related molecules. The objective of our initial work has been to obtain electron transport from the redox center of an encapsulated enzyme (making use of redox-active molecules or mediators) so as to measure the electrochemical response of the doped sol-gel to an external analyte.

INTRODUCTION.

In the sol-gel process, stepwise polycondensation of alkoxy silicate precursors is used for obtaining polymeric silica (1). Starting from a fluid sol, the transition to an amorphous gel results in a two-phase material in which the pores of the solid are filled with solvent. Continued physical evolution of the sol-gels through the processes of aging and drying yields inorganic materials with characteristic solid state properties. Aged gels are materials with solvent-filled pores while the ambient-dried xerogels are porous glasses with ~50% porosity by volume.

Porous silica sol-gel glasses containing immobilized biological molecules such as proteins and enzymes represent a new class of bioceramic materials (2). The porosity of the matrix is such that small molecules and ions can readily diffuse into the materials while the much larger proteins remain trapped in the silicate network. Thus, low molecular weight redox-active molecules (mediators) can be used to transfer electrons from the enzyme to the electrode. While the larger dimensions of the biomolecules restrict their movement through the gel, the low molecular weight mediators are mobile in the gel and can engage in electron-transfer events with the redox-active enzyme. Moreover, the mediators can also interact with the electrode surface, thereby establishing an indirect electron transport relay from the biomolecule to the electrodes. Based on this approach, it is possible to develop new types of "solid" state electrochemical

sensors which use the specificity of enzymes for catalysis of substrates or related molecules.

In the initial proof of concept experiments, sol-gel encapsulated alcohol dehydrogenase (ADH) was chosen as the bioactive molecule, while ferrocyanide ($Fe(CN)_6^{4-}$) ion was used as a mediator. An increase in current flowing through the system is observed due to release of electrons from the substrates and their subsequent transport to the electrode surface. The magnitude of the current corresponds directly with the concentration of the external substrate. These silica gels containing immobilized biorecognition molecules in combination with a mediator constitute an electrochemical device capable of serving as a biosensor.

This paper reports recent advances by our group directed towards testing the feasibility of ion mediated charge-transport in the aged gels with the intent of fabricating solid state electrochemical biosensor devices. Our primary objective has been to use mobile redox-active ions as carriers of charge and electrons across the material to make the gels ionically conducting. The sol-gel electrochemical sensors are based on electron transport from the redox center of an encapsulated enzyme with the use of redox-active mediators as electron transporters. We show that ions incorporated in the pores of the silicate matrix are effective towards achieving electron transfer relay from the redox-active sites of encapsulated biomolecules to the electrodes. The specific reactivities of these bioceramic materials towards substrate molecules can be used to obtain solid-state sensing elements. In this way, the electrochemical response of the doped sol-gel to an external analyte can be detected. The solid state nature of the gels is attractive for device applications. By using a modified sol-gel route, thin-film devices can also be fabricated.

EXPERIMENTAL.

Preparation of Sol-Gels.

The initial sol was prepared using tetramethyl orthosilicate (TMOS, Aldrich) as the precursor. To 15.27 g of TMOS in a beaker, were added 3.36 g of deionized water, and 0.22 g of 0.04 M HCl. The resultant mixture was kept ice-cooled in an ultrasonic bath and sonicated for 10-15 minutes. Upon completion of sonication a homogenous sol was formed and the sample was transferred to an ice-bath.

To form gels, this sol was mixed with an equal volume of the dopant solution. The gels were usually cast in polystyrene cuvettes. After gelation, the gels were sealed with parafilm and aged for a few days. The aged gels undergo a slight shrinkage and can be removed from the cuvettes after an aging period of two to three days. These aged gels were washed with deionized water to remove excess methanol formed during the sol-gel reaction. The samples were then immersed overnight in a saturated solution of ferrocyanide to diffuse ferrocyanide into the pores of the matrix.

Immobilization of ADH.

A buffered reaction medium was used for protein encapsulation. For immobilization of ADH, 0.1 M phosphate buffer (pH 8.01) was used to dissolve the protein. Lower pH values were found to give rise to aggregation of the protein. The best results were obtained by using a pH 8.01 phosphate buffer. Typically 5 mg/mL of the enzyme was used to make the stock solution. The enzyme solution (1 mL) was quickly mixed with the TMOS sol (1 mL) to form the gels. Gelation occurred rapidly (ca. 1 min.). Although the gels formed this way were transparent, they showed slight turbidity indicating minor aggregation effects. The gels were allowed to age for 2-3 days at 4°C. These aged sol-gels were then doped with appropriate mediators (ferrocyanide,) via immersion in a saturated aqueous solution of the mediator.

Thin-Film Samples.

The thin film samples were prepared using a modification of the buffered sol-gel route (3). The protein was immobilized in sol-gel thin-films deposited on oxidized silicon substrates patterned with interdigitated gold microelectrodes. These substrates were connected to a weighted float in a water reservoir whose drainage rate was controlled by a flow valve. The films were prepared by a dip coating technique where the substrates were withdrawn from a low viscosity sol containing the protein dopant. Excellent quality protein-doped thin-films that were homogeneous, crack-free, and adherent to the substrate were produced by this method.

The thin-films containing ADH were prepared by mixing a buffered solution of the enzyme with a preformed sol. Typically, a homogeneous sol was prepared by sonicating 15.27 g TMOS, 3.38 g. water and 0.22 mL of 0.04 M HCl for 10 minutes in a sonication bath containing a mixture of ice and water. The freshly prepared sol, methanol and a buffered solution of protein were kept in an ice-bath before mixing. For encapsulation of ADH, high pH conditions were employed. The TMOS sol (2.0 mL) was mixed in with 2.0 mL of methanol, and 0.5 mL of the ADH solution containing 25 mg/mL of the enzyme dissolved in 0.1 M phosphate buffer (pH 8.01). The films were dried in ambient for approximately 30 minutes. These dried films were then immersed in a saturated solution of ferrocyanide prior to electrochemical measurements.

Electrochemical Measurements.

The electrochemical measurements were performed using EG&G Princeton Applied Research model 173 potentiostat/galvanostat. A two-electrode configuration using ~1 cm^2 gold foil electrodes was employed for the measurements. The gels were kept in a polystyrene cuvette that was open at both the ends. The foil electrodes were physically pressed onto the ends of the gels to obtain electrical connections to the samples.

The gels containing the protein/mediator molecules were tested for electrochemical response by using the cyclic voltammetry subroutine of the electrochemical software. The sweeps were performed from +800 mV to -800 mV in 10 mVS^{-1} increments.

RESULTS AND DISCUSSION.

Mediated Electron Transport.

The isolation of a liquid phase in the pores of the solid gel matrix makes this constituent available for solution chemistry approaches which can be utilized for electron transport. Due to the interconnected porous structure of the gel matrix, the low molecular weight species are generally mobile. As opposed to ionic charge transport, redox-mediated electron transport does not accompany extensive mass transfer. The gels doped with redox-active molecules can be considered as solid electrolytes designed to have an electronic current flowing in an external circuit through connected metal electrodes. The external current is balanced by an ionic-electronic current in the internal circuit. In its simplest configuration, the experiment constitutes monitoring the electrical response of a gel sample by two gold metal electrodes placed at the ends of the monolith.

The solution phase in the porous gel network enables one to incorporate a variety of redox-active mediators. These mediators transport electrons from the redox center of an encapsulated enzyme. In these studies, the ferrocyanide ion $Fe(CN)_6^{4-}$ was used as a mediator. The two-electrode electrical response of the sol-gel sample containing only ferrocyanide ions (i.e. without encapsulated enzyme) is shown in Fig. I.

The sol-gels containing the ferrocyanide mediator show a linear ohmic response in the region of low overpotentials (Fig. I). The overall electron flow for the reaction in this case can be written as

$$\text{Cathode} \rightarrow \mathbf{Fe(CN)_6^{3-}} \rightarrow \mathbf{Fe(CN)_6^{4-}} \rightarrow \text{Anode}$$

where electron transport within the gel matrix is mediated by the $Fe(CN)_6^{4-/3-}$ couple. The equilibrium and the fast self-exchange reactions of the redox-couple ensure minimal concentration polarization. The redox-molecules are believed to transport electrons within the gel through a series of intermolecular outer-sphere electron transfer events. In other words, electron transport through the bulk of the material occurs by local migration and subsequent self-exchange between the two forms of the redox active anion. The electron transfer from one electrode to the other is thus quite similar to the valence-hopping mechanism observed in mixed-valence conductors. The electron-transport relay from the cathode to the anode through the material results in molecularly conducting silica sol-gel glasses.

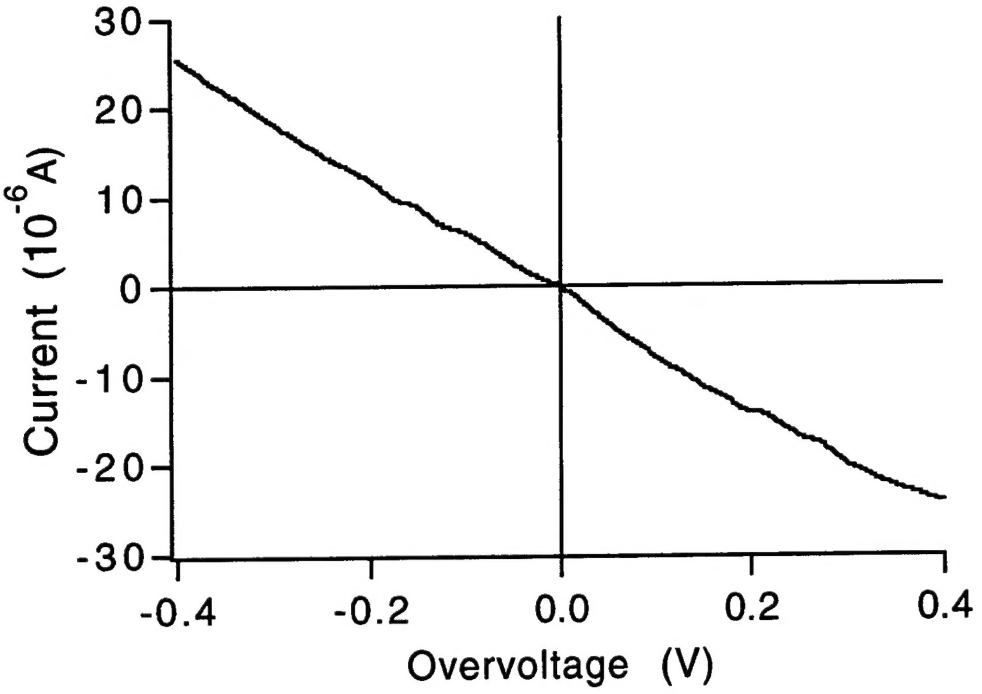
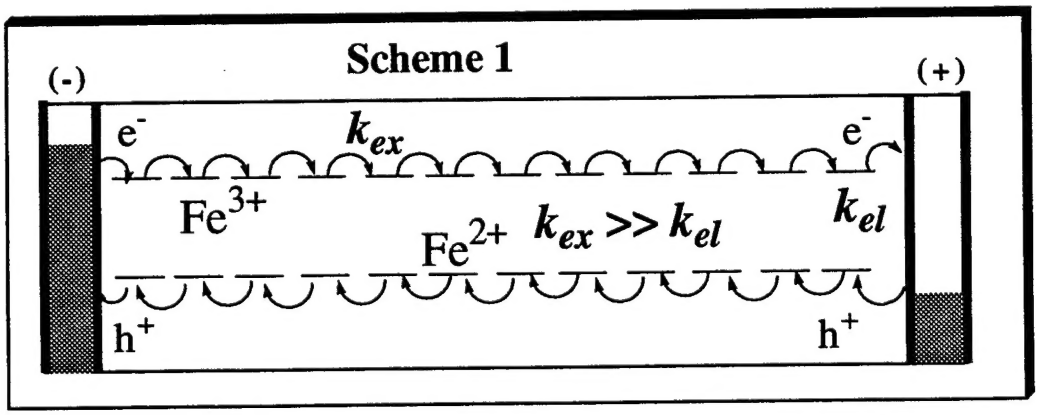


Figure I. Ohmic response in low-overpotential region showing mediated electron transport in silica sol-gel.

In the ferrocyanide-doped sol-gel system three primary steps take place. These include heterogeneous transfer of electrons from ferrocyanide to the anode, homogeneous transfer of electrons via self-exchange reactions, and heterogeneous transfer of electrons from the cathode to the ferricyanide molecule. The rate constant for the homogeneous electron transfer (k_{ex}) is usually very fast compared to the rate of electron transfer from the mediator to the electrode (k_{el}). The elementary events and the current associated with them can be represented as in Scheme 1.



A very interesting feature observed in this system is that the current response in this case is directly proportional to the concentration of the ferrocyanide present in the medium. At low overpotentials, the linear response observed is due to faster electron-transfer mediated by the ferrocyanide molecules. Any external stimulus (e.g. a biocatalytic reaction) which alters the ferro/ferricyanide ratio will change the current flowing through the system and can be measured analytically.

Electrochemical Biorecognition in Monolithic Sol-Gels.

The presence of an ionic-electronic current in the internal sol-gel matrix provides a simple way to couple the intrinsic conductivity with a biocatalytic reaction. In this way, the redox catalysis reaction can be monitored. If the catalytic reactions of the enzymes are coupled with this reaction then the rate and extent of catalysis can be monitored by the electrical response of the system.

The oxidation of ethanol catalyzed by ADH is an example of oxidation catalysis and electrons are released as a result of the following reaction:

$$C_2H_5OH \xrightarrow{ADH} CH_3CHO + 2e^-$$

As a result of the release of electrons into the system, an overall increase in current flowing through the sol-gel system is observed (Fig. II).

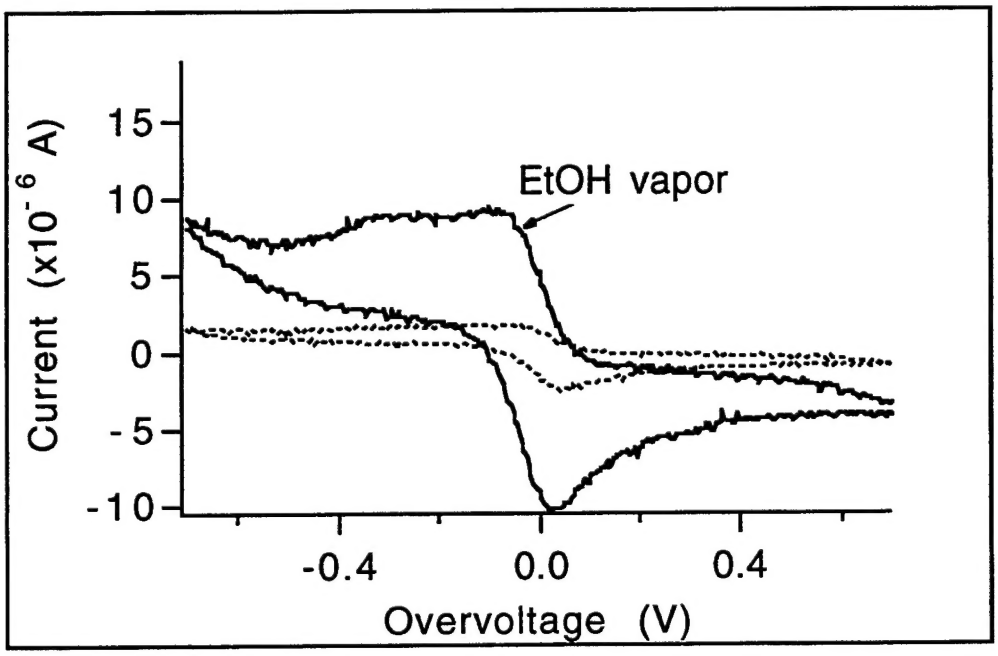


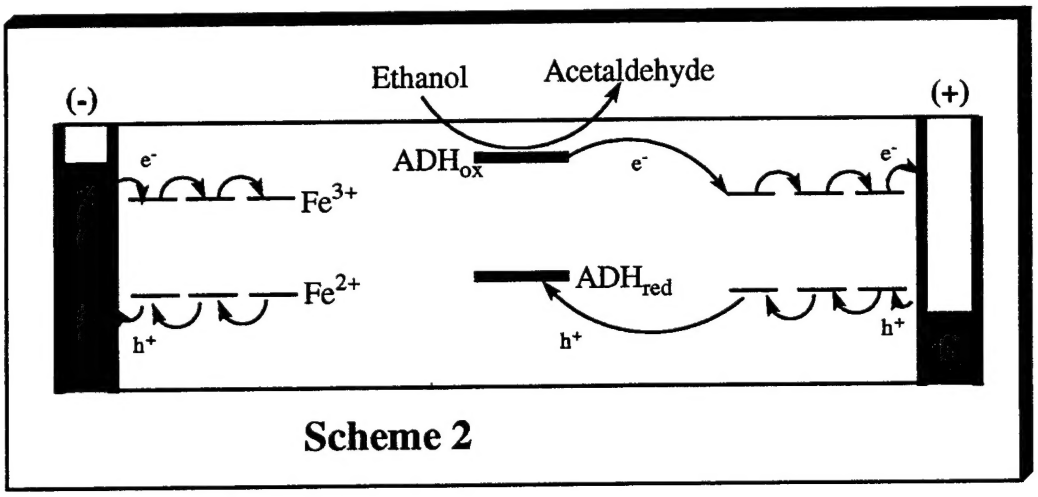
Figure II. Electrochemical response of ethanol biocatalysis by ADH in silica sol-gel.

Electrochemical Biosensor for Ethanol Vapor.

The electrical response of the sol-gel system was used in a biosensor device for detection of ethanol vapor according to the catalytic reaction (4):

$$CH_3CH_2OH + 2\,Fe(CN)_6^{3-} \xrightarrow{ADH} CH_3CHO + 2\,Fe(CN)_6^{4-} + 2\,H^+$$

The change in current flowing through the aged gel glasses was used as a measure of ethanol concentration. The experimental arrangement used for the device was based on exposing the porous gels to ethanol-water mixtures with a variable ratio of the alcohol. The advantages of this arrangement are twofold: 1) the porous structure of the sol-gel allows the ethanol molecules to diffuse into the system and 2) the use of a vapor phase analyte rather than a solution prevents leaching of the mediator molecules into the external solution. The retention of mediator ferrocyanide molecules in the system ensures a constant concentration. In this configuration, the electrical output of the system is related to the concentration of the ethanol substrate (Scheme 2).



Scheme 2

The performance of this alcohol biosensor was determined by exposing the glassy materials to different solutions that contained various water-ethanol ratios. The ethanol molecules in the vapor phase diffuse into the porous structure of the silica sol-gel materials and react with the encapsulated ADH. Upon reaction, the oxidation of the ethanol catalyzed by ADH generates electrons which are, in turn, carried by the ferrocyanide mediator and ultimately delivered to the gold electrodes. The current flowing through the system, therefore, serves as a measure of the concentration of the ethanol.

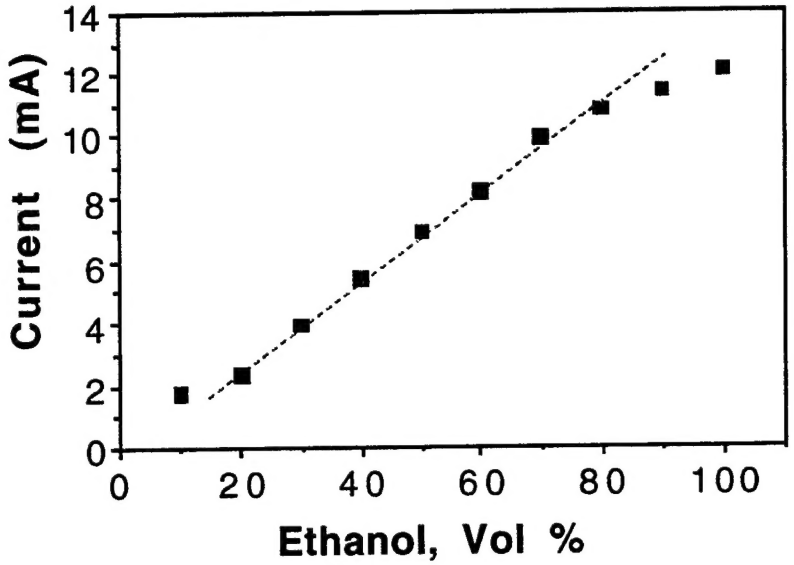


Figure III. Correlation of the current with concentration of the ethanol-water mixtures that were exposed to porous sol-gels.

The correlation curve for the ethanol vapor biosensor is shown in Fig. III. The current flowing through the system is correlated with the percentage of ethanol in the ethanol:water mixtures exposed to the gels. The number of ethanol molecules reacting with the immobilized ADH is directly proportional to the current measured across the electrodes. The curve shows a linear relationship between the range of ethanol concentrations from 20% to 80%. At higher concentrations, a saturation of the electrical signal was found to occur.

Electrochemical Biorecognition in Sol-gel Thin Films.

The electrochemical biorecognition of ethanol was also demonstrated in thin-films using a set of interdigitated gold electrodes. Analogous to monolithic samples, the oxidation of ethanol by the encapsulated ADH results in an increase in current. The shorter electrode separation provides good signal-to-noise while the use of thin films improves the response time of the device. It is important to mention that the current recorded by the thin-film based devices is on the same order of magnitude (~10 μA) as that using monolithic samples. The short distance (~100 μm) between electrodes in the interdigitated electrode pattern is responsible for the increased sensitivity.

CONCLUSIONS AND SIGNIFICANCE.

The present paper has shown that inorganic molecular ions migrate freely in aged silica sol-gel materials. These electroactive mediators are used to transfer electrons to and from the electrodes to the bulk of the material. The sol-gel materials containing an immobilized redox enzyme along with a mediator can be used as electrochemical biosensors. The porous nature of silicate glasses allows access to low molecular weight external analytes. Moreover, gaseous entities can also be detected. Sol-gel matrices are obtainable as thin-films, and it is shown that electrochemical bio-gel sensors using thin-films exhibit faster response times.

Traditionally, electrochemical biosensors have relied upon the covalent attachment of enzymes on the surface of an electrode (5). However, surface immobilization is a technologically complicated step. Furthermore, covalent immobilization is largely detrimental to enzyme reactivity, and therefore, limits its usage. On the other hand, sol-gel encapsulation is generally applicable to a wide variety of biological systems. The overall mechanism of detection is similar to solution electrochemistry. The overall mechanism of detection is based on changes in current flowing through the system. As such silica sol-gels containing encapsulated biorecognition molecules along with an electron mediator constitute an electrochemical device capable of serving as a biosensor element.

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